

Quantification of isoflavones in stems of faba bean (*Vicia faba* L.)

Cuantificación de isoflavonas en tallos de haba (*Vicia faba* L.)

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ABSTRACT

The isoflavone aglycones daidzein and genistein are phytoestrogens that ameliorate the symptoms of menopause and assist in the prevention of chronic diseases. These compounds have been studied mainly in soya but have also been detected in faba beans (*Vicia faba* L.). Since little information is available concerning the content of isoflavone aglycones in faba beans from Mexico, the present study aimed to establish the presence and concentrations of daidzein and genistein in stems of faba bean cultivars from the Mexican central plateau. Cultivars C-281, C-146, C-160, C-89, C-288, C-181, C-93 and C-Zac22 were grown under greenhouse conditions and 6 to 7 week-old stems were harvested. The presence of daidzein and genistein in these tissues was confirmed by thin layer chromatography using a solvent system containing toluene-ethyl acetate-acetone-formic acid (74:14.8:7.4:3.8). High-pressure liquid chromatographic analysis revealed significant differences ($p \leq 0.01$) in the concentrations of aglycones with levels of genistein in the range 0.30 to 0.85 mg kg⁻¹ dry weight (DW) and of daidzein, which was more abundant in all cultivars, present in the concentration range 34.92 to 59.98 mg kg⁻¹ DW. Stems of cultivars C-89, C-181, C-281 and C-146 were exceptional for their concentrations of isoflavones and may represent good alternative sources of phytoestrogens.

Keywords

daidzein • genistein • thin layer chromatography • high-performance liquid chromatography

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RESUMEN

Las isoflavonas agliconas, daidzeína y genisteína, son fitoestrógenos que benefician la salud, mejoran los síntomas de la menopausia y previenen enfermedades crónicas. Estos compuestos se han estudiado principalmente en soya (*Glicine max*), pero también se han detectado en habas (*Vicia faba* L.). Dado que hay poca información disponible sobre el contenido de isoflavonas agliconas en habas de México, el presente estudio tuvo como objetivo establecer la presencia y las concentraciones de daidzeína y genisteína en tallos de cultivares provenientes de la Meseta Central de México. Los cultivares C-281, C-146, C-160, C-89, C-288, C-181, C-93 y C-Zac22 se sembraron en un invernadero para la obtención de tallos entre 6-7 semanas posteriores a la siembra. Se comprobó la presencia de daidzeína y genisteína por cromatografía en capa fina usando un sistema de solventes de tolueno-acetato de etilo-acetona-ácido fórmico (74:14,8:7,4:3,8). Los resultados de la cromatografía líquida de alta resolución mostraron diferencias significativas ($p \leq 0,01$) con concentraciones para genisteína entre 0,30–0,85 mg kg⁻¹ y daidzeína entre 34,92–59,98 mg kg⁻¹ de materia seca, siendo este último el metabolito más abundante en todos los cultivares. Los cultivares C-89, C-181, C-281 y C-146 fueron sobresalientes en la concentración de isoflavonas, por esta razón, pueden ser una buena fuente de fitoestrógenos.

Palabras clave

daidzeína • genisteína • cromatografía en capa fina • cromatografía líquida de alta resolución

INTRODUCTION

Isoflavones (Is) are a subclass of flavonoids (15) with a distribution restricted predominantly to the subfamily Papilionoideae of the family Leguminosae (12). While species such as *Phaseolus vulgaris* L., *Cicer arietinum* L., *Lens culinaris* Medik. and *Vicia faba* L. are rich in isoflavones, most phytochemical studies have been conducted on *Glycine max* (L.) Merr. (soybean) in which these compounds are particularly abundant (3,18). The isoflavones exist in free form as aglycones or as glycosides conjugated with acetyl or malonyl groups (11). The aglycones daidzein (Da), genistein (Ge) and glycitein are the most studied because of their similarity to the estrogen 17 β -estradiol (5), and their reported beneficial effects on diseases such as chronic and cardiovascular disorders, osteoporosis, breast

cancer and prostate cancer, and on the symptoms of menopause (1). Da and Ge have been detected in a number of leguminous plants, although the amounts present vary considerably according to the species and tissue studied (8). For example, Kaufman *et al.* (1997) reported that stems of *V. faba* (faba bean) are a good source of Is with Da, which is the principal component, present in concentrations of up to 1 g kg⁻¹ dry weight (DW) depending on the cultivar analyzed.

Thin layer chromatography (TLC) is the method most commonly employed for the detection of Is (20), while high performance liquid chromatography (HPLC) is frequently used for the quantification of these compounds (3, 6). In order to improve the efficiency of aglycone extraction, the conjugated glycosides

present in plant material are typically hydrolyzed to the free forms that act as phytoestrogens in humans (4, 20).

In Mexico, little information is available concerning the presence and concentrations of Is in faba beans. Based on the hypothesis that the concentration of isoflavones varies with the cultivar, the objective of this study was to establish the presence and concentrations of Da and Ge in stems of eight cultivars of *V. faba* from the central plateau of Mexico.

MATERIALS AND METHODS

Plant material

Five cultivars of faba bean (C-146, C-160, C-89, C-181 and C-93) were collected in the state of Puebla, two (C-281 and C-288) in the state of Mexico and one (C-Zac22) in the state of Tlaxcala, all of which are located in the central plateau. Seeds of each cultivar were disinfected with 10% chlorine for 20 min and subsequently washed and left to soak in distilled water for 30 to 60 min until the seed coat softened. The prepared seeds were sown at a depth of 2 cm in a mixture of sandy soil and dry leaves (2:1) contained in 1 kg black plastic bags, with four seeds per bag and four bags per cultivar. The bags were maintained in a greenhouse and the seedlings were watered throughout the growing period. A first sowing was performed in February-March 2014 and plants were harvested six weeks after sowing (WAS), during growth stage 15 of leaf development (13), with subsequent TLC analysis of stem tissue for the presence of Da and Ge. A second sowing was performed in May-June 2015 and stems were collected seven WAS, during growth stage 16 of leaf development (13), with subsequent quantitative HPLC

analysis to establish the concentrations of Da and Ge present. Harvested stems were chopped manually and dried in a forced air oven at 40°C for 48 h, following which they were frozen in liquid nitrogen and ground in a mortar and pestle. The powder obtained was passed through a # 60 mesh (250 µm) sieve and stored at -20°C until required for analysis.

Extraction of isoflavones

The extraction of Is aglycones for TLC analysis was based on the method of Yu *et al.* (2000) with modifications. An aliquot (200 mg) of faba bean stem powder was weighed, mixed with 1 mL of 80% methanol, agitated for 30 s and centrifuged at 1160 *g* for 2 min at 4°C. The supernatant was transferred to an amber glass vial containing 3 mL of 1M hydrochloric acid and the mixture was incubated at 95°C for 2h, following which 1 mL of ethyl acetate was added and the mixture was stored at 4°C. The extraction of Is aglycones for HPLC analysis followed the method of Wang and Murphy (1994) with modifications. An aliquot (300 mg) of faba bean stem powder was weighed accurately and mixed with 5 mL HPLC ULTRA grade acetonitrile (J. T. Baker, product #9017), 1 mL of 0.5 M hydrochloric acid in ethanol and 0.05% 2,6- di-tert-butyl-4-methylphenol (BHT) (Sigma Aldrich, product # B1378). The mixture was homogenized at room temperature for 2h, filtered under vacuum and the filtrate taken to dryness in a Heidolph-Instruments model 4000 rotary evaporator at 37°C. The residue was dissolved in 1 mL of 80% HPLC grade methanol (J. T. Baker, product #9093), filtered through 0.45 µm Acrodisc® membrane filters (Pall Corporation, product #4556T) and stored at -40°C in amber vials for 24 h before analysis.

Analysis of isoflavone aglycones by TLC

Analyses were performed on 5 x 5 cm silica gel 60 F₂₅₄ plates (Sigma Aldrich, product #Z193275) eluted with toluene:ethyl acetate:acetone:formic acid (74:14.8:7.4:3.8 by volume) according to the method of Yuan *et al.* (2006) with modifications. Developed plates were exposed for 2 min to the vapor of a concentrated ammonium hydroxide solution (Meyer, product #0590) and viewed under ultraviolet light ($\lambda = 254$ and 302 nm) emitted by a UVP UVLMS-38 EL series UV Lamp (UVP, product #95025201). Samples of extracted aglycones were analyzed along with reference standards of daidzein (Supelco, product #16587) and genistein (Sigma Aldrich, product #G6776), dissolved separately or together in methanol at concentrations of 0.2 mg mL⁻¹, in order to identify the separated constituents.

Analysis of isoflavone aglycones by HPLC

The methodology proposed by Wang and Murphy (1994) was followed with some modifications. Analyses were performed using an Agilent Technologies Infinity 1260 HPLC system equipped with a diode array detector and a ZORBAX Eclipse XDB-C18 column (4.6 x 50 mm; 3.5 μ m particle size). Elution was with mobile phase A, comprising 0.1% glacial acetic acid in water, and mobile phase B, consisting of 0.1% glacial acetic acid in acetonitrile, commencing with 100% A for 2 min followed by a linear gradient from 95% to 65% over the remaining 38 min. Analyses were carried out at 25°C with a mobile phase flow rate of 0.5 mL min⁻¹, a sample injection volume of 10 μ L, and a detection wavelength of 260 nm. Stock solutions of reference standards Da and Ge containing, respectively, 40 and 1 μ g mL⁻¹ in 80% methanol were prepared and

diluted appropriately for the construction of calibration curves in the concentration ranges 10 to 20 μ g mL⁻¹ for Da and 0.01 to 0.3 μ g mL⁻¹ for Ge. Concentrations of Da (retention time 32 min) and Ge (retention time 37 min) in extracted samples were calculated and expressed in terms of mg kg⁻¹ DW of stem material.

Statistical analysis

Statistical analyses were performed on the concentrations of isoflavones determined by HPLC, considering the different cultivars of *V. faba* as the source of variation. On this basis, eight treatments with three replicates each were evaluated, giving a total of 24 samples. Data per treatment were analyzed with the aid of SAS/STAT statistical software, version 9 (16) employing a balanced completely randomized design (PROC ANOVA) and the Tukey test of comparison of means with the significance level set at $p \leq 0.05$.

RESULTS AND DISCUSSION

The eluent system employed in the TLC analysis of aglycones from stems of faba bean cultivars was of appropriate polarity for the separation of the reference standards Da and Ge (identified by letter E in figure 1, page 47). Visualization of the developed plates under UV light at 252 and 302 nm indicated R_f values of 0.27 for standard Da and 0.388 for standard Ge, equivalent to those (0.25 for Da and 0.38 for Ge) reported by Yuan *et al.* (2006). Bands with the same R_f values as Da and Ge, but with different intensities, were observed in the TLC profiles of extracts of faba bean cultivars. The results of this qualitative analysis confirms the presence of Da and Ge in faba bean plant stems harvested at six WAS, as documented previously by Kaufman *et al.* (1997).

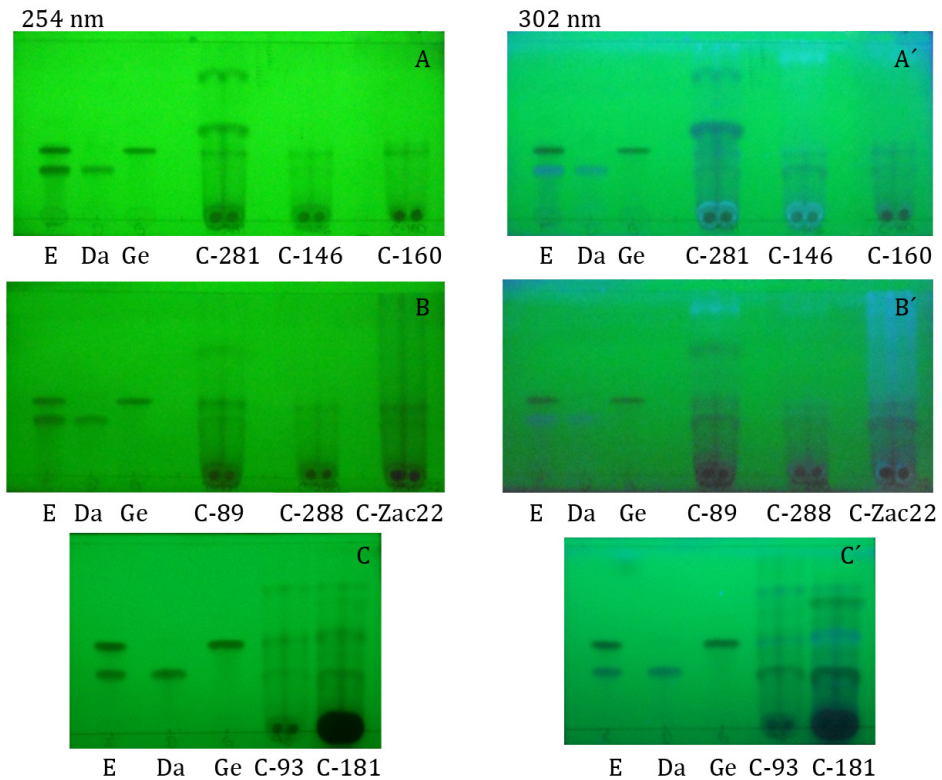


Figure 1. Images of thin layer chromatographic plates visualized under UV light at 254 nm (panels A - C) and at 302 nm (panels A' - C'). Lane identification: Da - standard daidzein ($R_f = 0.27$); Ge - standard genistein ($R_f = 0.388$); E - standards Da and Ge; C-281, C-146, C-160, C-89, C-288, C-Zac22, C-93 y C-181 - stem extracts of faba bean cultivars.

Figura 1. Imágenes de placas cromatográficas de capa delgada visualizadas bajo luz UV a 254 nm (paneles A - C) y a 302 nm (paneles A' - C'). Identificación del carril: Da - estándar de daidzeína ($R_f = 0,27$); Ge - estándar de genisteína ($R_f = 0,388$); E - estándares Da y Ge; C-281, C-146, C-160, C-89, C-288, C-Zac22, C-93 y C-181 - extractos de tallo de cultivares de haba.

HPLC analysis confirmed the presence of Da and Ge in the stems of all eight faba bean cultivars tested, although the overall mean concentration of Da was almost 100-times greater than that of Ge (table 1, page 48). The higher coefficient of vari-

ation obtained for Ge (22.46%) is attributable to the lower mean concentration values and their broader dispersion, likely because the quantification of this metabolite is more difficult and requires greater precision.

Table 1. Analysis of variance of daidzein and genistein concentrations in stems of *V. faba* cultivars.**Tabla 1.** Análisis de varianza de las concentraciones de daidzeína y genisteína en tallo de cultivares de *V. faba*.

Isoflavone	Mean (mg kg ⁻¹ DW)	CV [†] (%)	MS ^{††}
Daidzein (Da)	51.27	7.69	310.05**
Genistein (Ge)	0.48	22.46	0.09**
Total (Da + Ge)	51.76	7.72	310.52**

** Highly significant difference ($p \leq 0.01$). [†] Coefficient of variation. ^{††} Mean square.

** Diferencia altamente significativa ($p \leq 0,01$) [†] Coeficiente de variación. ^{††} Cuadrado medio.

The differences in mean concentrations of Da and Ge between cultivars were highly significant ($p \leq 0.01$). Such variation suggests that, because the cultivars were from different localities, they represent different genotypes and it is known that the concentration of Is can vary among genotypes of leguminous species (8). Interestingly, Kirakosyan *et al.* (2004) reported that the levels of Da and Ge in 10-day old faba bean seedlings also varied significantly among cultivars but did not show the same striking difference between aglycone concentrations as observed in the stem tissue analyzed in the present study.

The highest amounts of Da were detected in cultivars C-89, C-181, C-281 and C-146 with mean concentrations ranging from 58.29 to 59.98 mg kg⁻¹ DW, such levels being significantly different from those found in cultivars C-228 and C-93 that contained the lowest concentrations of the aglycone (table 2). Cultivar C-181 presented the highest level of Ge with a mean concentration of 0.85 mg kg⁻¹, a value that was significantly different from those of all other cultivars analyzed with the single exception of C-288. Cultivar C-181 also presented the highest mean concentration (60.74 mg kg⁻¹ DW) of aglycones Da and Ge taken together.

Table 2. Daidzein and genistein concentrations in stems of *V. faba* cultivars.**Tabla 2.** Concentración de daidzeína y genisteína en tallo de cultivares de *V. faba*.

Isoflavone	Cultivar							
	89	181	281	146	22	160	288	93
Daidzein (mg kg ⁻¹ DW)	59.98 ^a	59.88 ^a	59.20 ^a	58.29 ^a	53.44 ^{ab}	45.81 ^{ab}	38.77 ^c	34.82 ^c
Genistein (mg kg ⁻¹ DW)	0.43 ^{bc}	0.85 ^a	0.44 ^{bc}	0.32 ^{bc}	0.30 ^c	0.44 ^{bc}	0.64 ^{ab}	0.41 ^{bc}
Total (mg kg ⁻¹ DW)	60.42 ^a	60.74 ^a	59.64 ^a	58.61 ^a	53.75 ^{ab}	46.25 ^{bc}	39.41 ^c	35.23 ^c

Within each row, mean values bearing different letters are significantly different (Tukey, $p \leq 0.05$).

Letras diferentes por hilera indican diferencias estadísticas significativas (Tukey, $p \leq 0,05$).

The phytoestrogens Da and Ge have been widely studied with respect to their potential benefits to health. Most studies have been carried out with extracts of *G. max*, and only a few have focused on other legumes. However, Da and Ge are also found in green lentil seeds (*L. culinaris*) in combined concentrations of 12.70 - 14.60 $\mu\text{g kg}^{-1}$ DW and in red beans (*P. vulgaris*) at 19.00 - 24.10 $\mu\text{g kg}^{-1}$ DW (10), but these values are substantially lower than those reported herein (35.23 - 60.74 mg kg^{-1} DW) for stems of faba beans. It should be noted, however, that the levels of Is in legumes can vary according to environmental conditions, susceptibility to pathogens and genotypes studied (8). For example, Kirakosyan *et al.* (2004) analyzed seedlings of 24 cultivars of *V. faba* from different countries and found combined levels of Da and Ge that ranged up to 19.30 mg kg^{-1} DW, although in some cultivars these metabolites could not be detected.

Anderson *et al.* (1999) reported that Is from soya would exert beneficial effects on tissues in menopausal women at doses of 60 - 100 mg day^{-1} for cardiovascular tissue and at more than 60 mg day^{-1} for bone tissue, while for climacteric symptoms a dose of approximately 20 mg day^{-1} would be required. Stems of faba bean plants are not currently documented as edible tissues and they are generally discarded, incorporated into the soil or harvested and used for animal feed. However, results from the present study show that faba bean stems, particularly those from cultivars C-89, C-181, C-281 and C-146, contain Da and Ge at levels that suggest their potential use as sources of phytoestrogens that could be incorporated into functional foods or nutraceutical products (17). The study and identification of the isoflavone content in faba bean cultivars are of importance not only to Mexico, but also to other countries where *Vicia faba* is cultivated, in order to

have a local source of plant materials with a high phytochemicals content. In a similar way as in some species of Brassicaceae, in which recommendations are made for their consumption to take advantage of the bioactive phytochemicals present throughout the year in Argentina (7).

The finding that Da is present in faba bean stems at significant concentrations is important because intestinal bacteria can transform this phytoestrogen into equol and O-desmethylangolensin. Both of these metabolites display greater biological activities than their precursor compounds (2), while there is some evidence that equol may be associated with the prevention of certain types of cancer (14). Although the benefits of these phytoestrogens have been documented, it is also well known that they can cause toxicological alterations in the endocrine system and further studies in this area are required since the effects on humans appear to depend on age, state of health and specific intestinal microbiota of each individual (14).

CONCLUSIONS

Daidzein and genistein were found to be present in the stems of all cultivars of *V. faba* collected from the central plateau of Mexico. Although the concentrations of these aglycones varied markedly amongst the cultivars, Da was the predominant metabolite in all samples analyzed. Under the greenhouse conditions employed in the study, the content of Da + Ge in faba bean stems was in the range 35.23 to 60.74 mg kg^{-1} DW. Cultivars C-89, C-181, C-281 and C-146 were outstanding for their isoflavone concentrations and represent good sources of phytoestrogens. It is concluded that faba bean stems could be an attractive option for isoflavone extraction and use in nutraceutical products.

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